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14. ABSTRACT

This project is progressing excellently and remains on schedule across research sites as defined by the Statement of Work. From studies completed to date, our major findings are that (a) morphine-induced increases in extracellular dopamine in the Nucleus Accumbens (NAc) shell is suppressed by microinjection of the toll like receptor 4 (TLR4) antagonist LPS-RS into either the NAc or the ventral tegmental area (VTA). Intriguingly, suppression of NAc dopamine in response to cocaine is also observed by NAc or VTA LPS-RS. This is exciting as it challenges the broadly held view that the major effect of cocaine is on dopamine transporters in the VTA. Given this intriguing result, a control study was undertaken to ensure specificity of the results obtained. Specificity was observed since LPS-RS had no effect on dopamine released in response to intra-VTA neurotensin. Multiple additional Aims and SubAims have begun and significant progress anticipated in the coming project period.

15. SUBJECT TERMS
toll like receptor 4 (TLR4); TLR4 agonists *non*-opioid (+)-naloxone and (+)-naltrexone; drug abuse; glial activation; therapeutic approach to treating drug abuse; opioids; cocaine

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Introduction:

The reinforcing and addictive properties of abused drugs, such as morphine and cocaine, are largely attributed to their ability to activate the mesolimbic dopamine pathway, resulting in increased extracellular dopamine in the nucleus accumbens shell (NAc). Under normal circumstances, the ventral tegmental area (VTA) strictly regulates dopamine levels within the NAc. Morphine and cocaine are known to interact with the central nervous system to produce distinctly different effects, both subjectively and physiologically; yet each drug is capable of increasing extracellular dopamine. To date, the bulk of research efforts have focused on how each drug interacts with its respective receptor targets on neurons. However, recently there has been more attention paid to the glial cells of the brain and how they might be involved in neurobiological mechanisms underlying the effects of drugs of abuse.

Morphine is known to act at mu-opioid receptors, which are located on neurons, both to produce its analgesic and rewarding/reinforcing effects. However, opioids—whether through prescription-based use to control pain or in abuse/illicit settings—have many unwanted side-effects, including tolerance (both for reward and pain relief), addiction, and severe withdrawal symptoms, among many others. Our laboratory recently published data demonstrating that morphine exerts many of these effects through activation of glial cells. Morphine-induced glial activation results in a powerful pro-inflammatory cascade, including the release of pro-inflammatory cytokines such as IL-1β and TNFα. These cytokines, and other pro-inflammatory molecules are neuroexcitatory and have the ability to interact with and effect neuronal functioning. Furthermore, we identified that the receptor through which morphine was inducing glial activation is Toll-Like Receptor 4 (TLR4), an innate immune receptor responsible for detecting pathogens. After showing that blockade of the TLR4 receptor improved morphine's analgesic properties and attenuated analgesic tolerance, we began to investigate the roll TLR4 signaling might have on morphine reinforcement. Preliminary studies demonstrated that systemic antagonism of TLR4 resulted in a blockade of both conditioned place preference (CPP) and self-administration, as well as a suppression of morphine-induced DA increase in the NAc. As intriguing as this finding is, it offers very little insight as to whether or not TLR4 signaling is directly involved in the mesolimbic pathway response to opioids, or whether there is some other less selective explanation for this phenomenon. Current pharmacological treatments for opioid addiction/abuse tend to be only effective and helping with decrease of illicit use, but require the continued use of a maintenance opioid, with lower abuse potential, that is costly and limited in success. Considering the increasing reports of opioid abuse, particularly abuse of prescription opioids, investigation into other treatment targets is of particular interest. TLR4 is an extremely interesting target to investigate, as early studies indicate that blockade of this receptor seems to preserve the desired effects of

opioids (pain-relief) while diminishing unwanted effects (analgesic tolerance and reward/reinforcement leading to addiction/abuse) .

Morphine is thought to exert most of its mesolimbic dopamine effects through actions in the VTA, where it disinhibits, or "turns down", VTA control of dopaminergic projections, allowing for more dopamine release in the NAc. However, the prevailing hypothesis is that cocaine induces an increase of dopamine in the NAc through blockade of dopamine transporters (DAT), re-uptake and clearance of dopamine from the synapse, resulting in an increased concentration of dopamine. In particular, research has focused on cocaine blockade of DAT in the NAc. However, medication development approaches focusing on disrupting inhibition of DAT by cocaine are largely unsuccessful, and cocaine abuse remains widespread, highly problematic, and extremely difficult to treat. We have recently demonstrated that cocaine also interacts with the Toll-Like Receptor 4 (TLR4) complex and that this interaction may be an important contribution to the neurobiological effects of cocaine underlying reinforcment, leading to subsequent abuse and addiction. Systemic interruption of TLR4-cocaine signaling results in a blockade of models of drug reinforcment including cocaine-induced dopamine increases in the NAc, and a suppression of cocaine conditioned place preference, self-administration, and reinstatement to self-administration. These findings suggest that TLR4 signaling may be critical to both the reinforcing effects of cocaine and opioids such as morphine.

The purpose of this grant is to further investigate this remarkable finding finding to better understand the role of TLR4 signaling in drug reward/reinforcement in order to determine the potential clinical utility of this previously unknown mechanism. These results not only fundamentally alter and expand current understanding of the neurobiological mechanisms underlying drug reinforcement, but also offer a new potential target for medication development to treat cocaine abuse.

Body:

Task 1: **Obtain approval from the Institutional Animal Care and Use Committee at University of Colorado Boulder for work to be done in the Watkins-Maier lab (University of Colorado-Boulder), Bachtell lab (University of Colorado-Boulder) and Katz lab (National Institute on Drug Abuse Intramural Research Program).**

Task 1 has been completed on time for all sites and animal research is in progress.

Task 2: **Receive (+)-naltrexone, as needed across the project period, from Dr. Kenner Rice (National Institute on Drug Abuse Intramural Research Program).**

Task 2 is successfully undertaken; all (+)-naltrexone needed to date has been received as committed by Dr. Kenner Rice

With accomplishment of Tasks 1 and 2, Milestone 1 was successfully achieved.

Watkins-Maier Research Lab:
Task 3 Aim 1A: Is morphine or cocaine CPP blocked by microinjecting a TLR4 antagonist (LPS-RS) into VTA or NAc shell?

Task 3 is in progress in the Watkins-Maier lab.

We have established the ideal coordinates for bilateral VTA cannula placements and those coordinates have been verified using histology in rat brains. Currently, pilot studies to define ideal microinjection drug dosing and timing are underway. The aim of this task is to assess a behavioral reflection of drug reward, which has been shown to correlate with extracellular dopamine changes in the NAc. Although the CPP studies (Aim 1A) were originally proposed to precede the microdialysis studies (Aim 1B, Task 4 below), the studies proposed in Aim 1B (Task 4) were undertaken first as the results from those studies will streamline the development of exact scientific design (for example, physiologically and neurochemically relevant drug dosing) for both SubAims thereby allowing all the projects to be completed in a more timely, efficient manner.

Task 4 Aim 1B: Are cocaine-induced increases in extracellular DA in NAc shell blocked by microinjection of LPS-RS into the VTA or NAc shell?

Task 4 is near completion in the Watkins-Maier lab.

We have recently found evidence that cocaine interacts with TLR4, a previously unidentified cocaine target in the central nervous system. However, it was unknown whether or not cocaine interactions with TLR4 were relevant to the reward processing regions of the brain, particularly the mesolimbic dopamine pathway, known to be critically involved in the rewarding effects of cocaine. This study was undertaken in order to further investigate the potential involvement of these brain regions, namely the dopaminergic projections from the VTA terminating in the NAc. Preliminary data indicate that TLR4 signaling in both regions appears to contribute to cocaine-induced increase of dopamine. However, our findings with the VTA in particular piqued our interest because they are somewhat exceptional and unconventional.

We found that intra-VTA administration of the TLR4 antagonist, LPS-RS, blocks cocaine induced dopamine increase in the NAc (**fig 1**). The implications of these data are very intriguing in two respects. The first is in regard to the traditional view on how cocaine exerts its' rewarding effects. Cocaine is believed

to act predominantly in the NAc, through DAT blockade. However, our data indicate that cocaine's actions in the VTA at TLR4 are critically involved in the ability of cocaine to induce DA increases in the NAc. The second is that this work essentially validates that TLR4 does indeed function in the meslolimbic

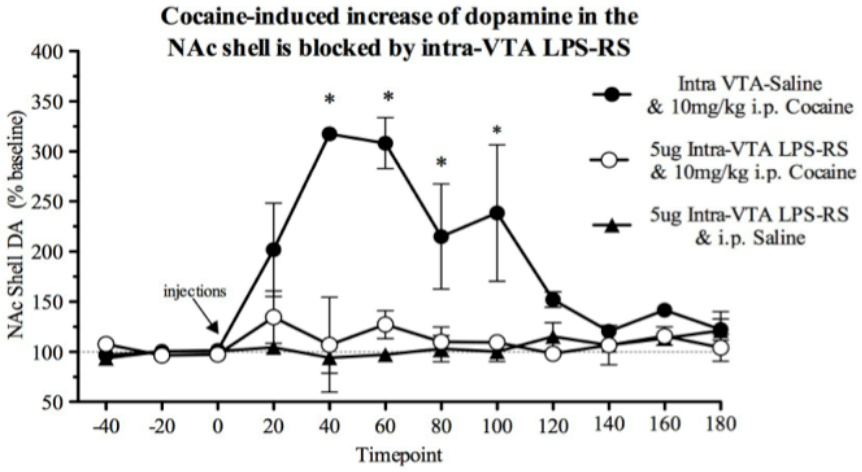

Figure 1: Rats were surgically implanted with microdialysis guide cannulae into the NAc shell and an ipsilateral microinjection cannula into the ventral tegmental area. Following a 7 day recovery period, rats were transported to the testing room for habituation, microdialysis probes were inserted and artificial cerebrospinal fluid flowed overnight. The next morning, after 3 baseline samples were collected, rats received either a 1uL injection of 5µg LPS-RS or 1µL sterile saline 10 minutes prior to an intraperitoneal (i.p.) injection of 10mg/kg cocaine or equivolume sterile saline. Samples were collected every 20 min for the duration of the study. Samples were analyzed using High Performance Liquid Chromatography (HPLC) to quantify dopamine concentrations. Analysis by two-way ANOVA revealed a statistically significant interaction ($p<0.0001$) of time and treatment. A bonferoni post-hoc test revealed that systemic cocaine paired with intra-VTA resulted in a significantly higher concentration of dopamine than the intra-VTA LPS-RS/systemic saline group ($p<0.01$) as expected. Intra-VTA LPS-RS administration blocked the dopamine increases caused by systemic cocaine, and cocaine concentrations in that treatment group are not different from the saline control group ($p>0.5$). (n= 5-7 per group)

dopamine pathway as a nonclassical cocaine receptor.

Because these results were so striking, the potential for other confounding explanations arose and became a concern to our research group. One concern was that TLR4 antagonism could be somehow "shutting down" or interfering with normal dopaminergic cell functioning. Even though we included a control group where rats received LPS-RS microinjections into the VTA, and in this case there was no effect on basal dopamine levels, there was still the potential the LPS-RS was interfering with dopaminergic neurons ability to become activated. In order to address this concern, we conducted a control study where rats received intra VTA injections LPS-RS following by a microinjection of the endogenous peptide, neurotensin. Neurotensin has been shown to increase dopamine concentrations

in the NAc when injected into the VTA, but being endogenous in origin, is very unlikely to interact with the TLR4 complex. Our results indicated that LPS-RS did not interfere with the ability of neurotensin to induce an increase of dopamine in the NAc (**fig 2**).

As mentioned previously, preliminary studies to date suggest that intra-NAc LPS-RS administration also attenuates cocaine-induced DA increases in the

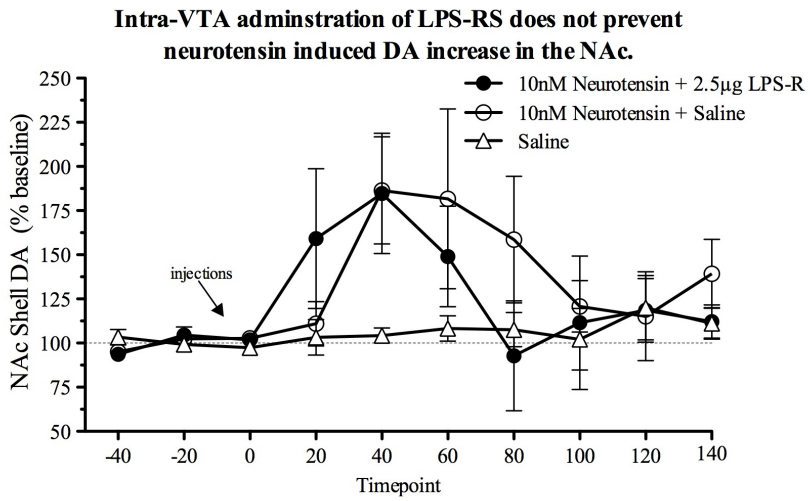

Fig 2: Experiment was conducted as described in figure 1, except that rats received a 10nM intra-VTA injection of neurotensin 10 minutes following a 1uL intra-VTA microinjection of 5µg LPS-RS. Although preliminary statistical analysis indicates that there is an effect of treatment, the n for each group is currently not equal (n=2-6) and for correct and appropriate analysis, the groups will be filled-in and statistically analyzed at that time.

NAc (**fig 3**). We are currently in the process of adding final numbers to each treatment group and decoding and analyzing HPLC data from those studies and are nearing completion on this task.

Task 5 Aim 3A&B: Which cell types(s) (microglia, astrocytes, oligodendrocytes, endothelial cels, neurons) express TLR4 in VTA and/or NAc, basally vs. after chronic morphine/cocaine?

We have begun pilot studies to define the optimal approach as our tests of commercially available anti-TLR4 antibodies have proven them to not be trustable. When run on western blots to examine selectivity, far too many errant bands (non-specific staining of non-TLR4 proteins) show up for us to trust the reagents tested to date. We will continue to explore anti-TLRs as others become available.

Upon recognizing the extent of problems with commercially available TLR4 antibodies, we have begun exploring the potential of antibodies directed against MD2, the critical co-receptor of TLR4 that heterodimerizing with TLR4 to induce signaling. Our pilot studies are suggestive of dense MD2 staining in naïve brains and reduction of MD2 staining 90 min after lipopolysaccharide (classical TLR4 agonist). Our preliminary interpretation is likely obscuring of the antibody binding

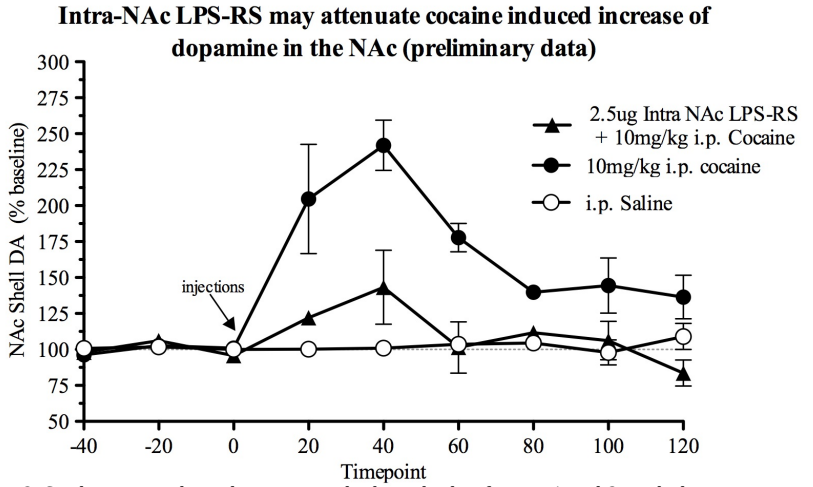

Fig 3: Study was conducted as previously described in figures 1 and 2, with the exception that an Eicomm omni-probe guide canula, allowing for both microinjections and microdialysis in the same brain region, was implanted and aimed at the NAc shell. During the experiment, rats received 2.5 µg intra-NAc LPS-RS in 0.5 µL and 10 minutes later received an interperitoneal injection of 10mg/kg cocaine. These data have not yet been analyzed because of low group n (n=3-4) but are thus far very promising and suggest that intra-NAc LPS-RS may attenuate cocaine induced dopamine increase in the NAc.

site by lipopolysaccharide or potentially internalization of MD2/TLR4.

We are also developing the means of rapid isolation of various cell types from discrete brain sites via flow cytometry with cell collections. This approach, which we have begun pilot studies of, with promising early results, would allow us to then analyze expression of MD2 and TLR4 by RT-PCR to reach the goal of the project.

Bachtell Research Lab:
Task 3 Aim 2A. Self- administration: Is cocaine reinforcement inhibited by systemic dosing with the TLR4 signaling inhibitor (+)-naltrexone? If so, is

cocaine reinforcement inhibited by microinjecting LPS-RS into VTA or NAc shell?

Experiment 1: Acquisition of Cocaine Self-Administration
Methods: This experiment is assessing the direct effect of TLR4 receptor antagonism on acquisition and maintenance of cocaine self-administration. We also tested the indirect effects of TLR4 antagonism on subsequent cue- and cocaine-induced reinstatement in the animals run to date. Osmotic minipumps (14-day 2mL) were filled with either (+)naltrexone (15mg/kg) or sterile water and implanted two days prior to the start of self-administration. Animals were then permitted to self-administered cocaine (0.5 mg/kg/infusion, iv) two hours per day over the next 17 days. After the self-administration session on day 13, the minipumps were removed and the animals continued with cocaine self-administration for an additional 4 days. The animals then underwent six days of extinction training followed by cue and then cocaine-induced reinstatement (15mg/kg, ip), where they were allowed to lever-press for two hours.

Results: Chronic administration of 15 mg/kg/day (+)naltrexone revealed no change in the acquisition or maintenance of intravenous cocaine self-administration without prior lever-press training. Thus, (+)naltrexone failed to affect the acquisition of self-administration during the first week of testing, and cocaine intake stabilized at similar levels in all groups during the second week of self-administration. There was a trend for (+)naltrexone to increase cocaine intake following (+)naltrexone minipump removal compared to vehicle minipump removal. After the last self-administration session, rats were progressed to extinction conditions to identify the indirect effects of (+)naltrexone administration on cocaine seeking. Animals administered (+)naltrexone during cocaine self-administration exhibited significant reduction in drug-paired lever responding compared to controls during the first extinction test. These data suggest that TLR4 inhibition during cocaine intake may decrease subsequent drug seeking that is indicative of drug craving. Following extinction, discrete cues that previously were paired with cocaine injections showed similar abilities to reinstate cocaine seeking in both (+)naltrexone and control groups. Likewise, the administration of 15 mg/kg cocaine produced reinstatement to cocaine seeking similarly in both groups as well. Together, these data suggest that TLR4 inhibition with (+)naltrexone administration during cocaine self-administration does not affect acquisition and maintenance of cocaine intake, but may reduce subsequent cocaine seeking. Futures studies will be directed at assessing a higher dosing of (+)naltrexone (30 mg/kg) during cocaine self-administration to determine whether these effects can be exacerbated with more potent TLR4 inhibition.

Experiment 2: Fixed/Progressive Ratio
Methods: This experiment is assessing the effects of TLR4 antagonisms on cocaine reinforcement using a progressive ratio schedule. In the animals run to

date, the animals were allowed to self-administer cocaine (0.5 mg/kg/infusion, iv) for two hours per day on a fixed ratio 1 (FR1) schedule for six days, and were then moved to a fixed ratio 5 (FR5) schedule for four days. Osmotic minipumps (7-day 2mL) were filled with either (+)naltrexone (15mg/kg) or sterile water and implanted after the last day of self-administration on FR5 (one day prior to the start of the progressive ratio schedule). The animals self-administered cocaine on progressive ratio for five days, and the pumps were removed following the final self-administration session. The animals then underwent nine days of extinction training followed by two-hour cue and cocaine-induced reinstatement (15mg/kg, ip) sessions.

Results: The progressive ratio schedule of reinforcement is the hallmark procedure used to identify the reinforcing efficacy of drugs of abuse by assessing the amount of effort an animal is willing to exert to obtain cocaine reinforcement. Chronic administration of 15 mg/kg/day (+)naltrexone during progressive ratio testing produced no change in either the number of cocaine infusions delivered or the final ratio completed (breakpoint) to earn a cocaine infusion. These findings suggest that 15 mg/kg/day (+)naltrexone does not influence cocaine reinforcement mechanism. Future work will assess the effects of 30 mg/kg/day (+)naltrexone on progressive ratio responding.

Task 4. Aim 2B. Is cocaine reinstatement to drug seeking blocked by systemic (+)-naltrexone? If so, is cocaine-induced reinstatement of drug seeking inhibited by LPS-RS microinjection into the VTA or NAc shell?

Cocaine reinstatement
Methods: This experiment will assess the effect of acute administration of (+)naltrexone on cocaine-induced reinstatement. The animals were allowed to self-administer cocaine (0.5 mg/kg/infusion, iv) for two hours per day for fifteen days. They then underwent extinction training for five days where lever presses were not reinforced. During reinstatement testing, the animals first had a two-hour extinction session immediately followed by a pretreatment of two injections of (+)naltrexone (15mg/kg, s.c.) or saline vehicle spaced thirty minutes apart. After the second (+)naltrexone injection, the animals received either a cocaine (15 mg/kg, ip) or saline vehicle prime. Non-reinforced lever pressing (active and inactive) was recorded during the two-hour session.

Results: This study is ongoing. All animals were trained to self-administer cocaine over 3 weeks. Lever responding was then extinguished in daily sessions where lever responding no longer produced the delivery of a cocaine infusion. After lever responding was extinguished to criterion, responding was reinstated by the administration of 15 mg/kg cocaine preceded by an acute (+)naltrexone (1.5, 3.75, 7.5 or 15 mg/kg, ip) or vehicle injection. Cocaine induced significant reinstatement of cocaine seeking. In animals tested to date, the data suggest we will likely find that cocaine restatement may well be reduced by the pretreatment

of 15 mg/kg (+)naltrexone. This study will continue during Year 2 as indicated by the approved SOW.

LPS-RS microinjections

Methods: These experiments will assess the effect of TLR4 antagonism specifically in the ventral tegmental area (VTA) or nucleus accumbens shell (NAcSh) on cocaine-induced reinstatement. The planned methods are as follows. Prior to the start of self-administration, the animals were implanted with both an intravenous catheter and guide cannula directed into either the NAcSh (Exp. 4) or the VTA (Exp. 5). After recovery from surgery, the animals self-administered cocaine (0.5 mg/kg/infusion, iv) for two hours per day for fifteen days. Animals then underwent extinction training in daily two-hour extinction sessions for nine days. During reinstatement testing, the animals first had a two-hour extinction session immediately followed by a microinjection pre-treatment of LPS-RS (5ug/side) or saline vehicle followed by a cocaine (15mg/kg, i.p.) or saline vehicle prime. Non-reinforced lever pressing (active and inactive) was recorded during the two-hour session.

To date, we have become proficient in the cannulae implantations in VTA and NAc to match our injection sites with those of the Watkins-Maier laboratory to ensure that results can be directly compared. Pilot studies are underway, with significant progress anticipated in the coming year.

Katz Research Lab:

Task 3. Aim 1B. Are morphine-induced increases in extracellular DA in NAc shell blocked by microinjection of LPS-RS into the VTA or NAc shell?

This Task is in progress.

The goal of this task is to explore the effects of site-specific TLR4 antagonism on morphine-induced DA increases in the NAc. We have established stereotaxic coordinates and surgeries have been perfected. Pilot studies have been run to identify the ideal dosing and timing of LPS-RS and morphine. Preliminary data imply that either intra-VTA or intra-NAc blockade of morphine-TLR4 signaling results in a suppression of morphine-induced dopamine in the NAc. Although preliminary, the implications of this data should it hold course are very interesting. Once again, TLR4-signalling may be involved in morphine reward in *both* the VTA and the NAc, although the predominate hypothesis is that morphine exerts its reinforcing effects through actions in the VTA alone. Based on the control studies run in Task 4 Aim 1A, the cocaine experiments, we know that LPS-RS is not interfering the dopaminergic cell functioning and that those neurons are still capable of being activated to produce elevated dopamine concentrations in the NAc, as shown when neurotensin in administered into the VTA following LPS-RS microinjection (fig 2). Data collected to date is extremely promising and it appears that VTA blockade of TLR4 may indeed suppress morphine induced increases of dopamine in the NAc (fig 4). However, at this point (n= approximately 3-6) larger group numbers are needed in order to identify

outliers and run statistical testing of the significance of the effect. Those experiments are currently underway, and we are approaching completion of this part of the task.

The second part of the task involves investigating whether or not TLR4 signaling within the NAc is involved in morphine induced increases of NAc dopamine. This study is in it's early stages. Pilot studies have been conducted to determine ideal LPS-RS doses for this paradigm. The experiment is currently underway, with some samples undergoing HPLC analysis, and more groups being run, and animals on order to fill each group.

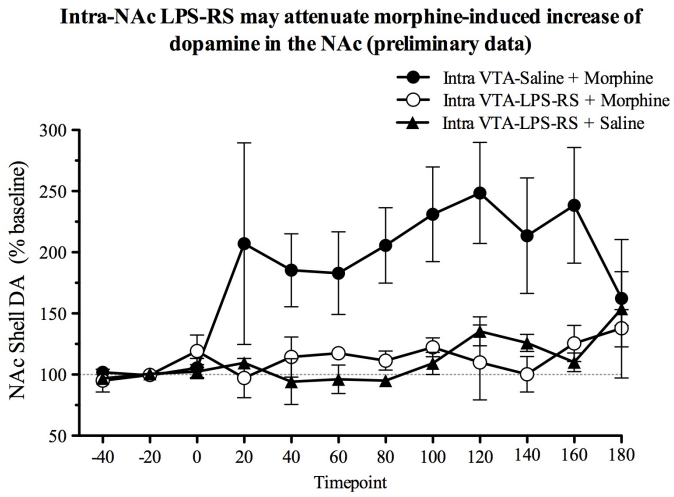

Fig. 4: Experiment was conducted as described in figure 1 except that rats received either a 1µL intra-VTA injection of 5µg LPS-RS or sterile saline immediately prior to a subcutaneous administration of either 6mg/kg morphine or equi-volume sterile saline. Although preliminary statistical analysis indicates that there is a highly significant effect of treatment, the n is currently neither equal across groups (n=2-6) nor large enough to be considered reliable and accepted by the field. For correct and appropriate analysis, the groups will be filled-in and statistically analyzed at that time.

Task 4. Aim 2A. Self-administration: Is morphine reinforcement inhibited by systemic dosing with the TLR4 signaling inhibitor (+)-naltrexone? If so, is morphine reinforcement inhibited by microinjecting LPS-RS into VTA or NAc shell?

This study has recently begun. To date, procedures generally followed those described by Hiranita et al. (2009). Sprague-Dawley rats (Taconic Farms, Germantown, New York) weighing approximately 300 g at the start of the study, serve as subjects. Subjects are acclimated to a temperature- and humidity-controlled vivarium for at least one week with a 12:12-h light:dark cycle (lights on at 07:00 hours) during which food (Scored Bacon Lover Treats, BIOSERV,

Frenchtown, NJ) and tap water were available at all times. After acclimation, body weights are maintained at approximately 320 g by adjusting the daily food ration with water remaining available at all times in the home cages. Care of the subjects is in accordance with the guidelines of the National Institutes of Health and the National Institute on Drug Abuse Intramural Research Program Animal Care and Use Program, which is fully accredited by AAALAC International.

Subjects are surgically prepared under anesthesia (ketamine 60.0 mg/kg, i.p. and xylazine 12.0 mg/kg, i.p.) with a chronic indwelling catheter in the right external jugular vein. The catheter exits the subject at the mid-scapular region of its back. Catheters are infused daily with 0.1 ml of a sterile saline solution containing heparin (30.0 IU/ml) and penicillin G potassium (250,000 IU/ml) to minimize the likelihood of infection and the formation of clots or fibroids. All animals are allowed to recover from surgery for approximately seven days before drug self-administration studies were initiated.

Experimental sessions are conducted daily with subjects placed in operant-conditioning chambers (modified ENV-203, Med Associates, St. Albans, VT) that measure 25.5 x 32.1 x 25.0 cm that are enclosed within sound-attenuating cubicles equipped with a fan for ventilation and white noise to mask extraneous sounds. On the front wall of each chamber were two response levers, 5.0 cm from the midline and 4.0 cm above the grid floor. A downward displacement of either lever with a force approximating 0.20 N defines a response, and always activates a relay mounted behind the front wall of the chamber producing an audible “feedback” click. Six light-emitting diodes (LEDs, three yellow and three green ones) are located in a row above each lever. A house light is located at 25 cm above the grid floor (near the ceiling) at the center of the front wall of the chamber. A receptacle for the delivery of 45-mg food pellets via a pellet dispenser (Med Associates, Model ENV-203-20), is mounted on the midline of the front wall between the two levers and 2.0 cm above the floor. A syringe infusion pump (Model 22, Harvard Apparatus, Holliston, MA) is place above each chamber delivered injections of specified volumes from a 10 ml syringe. The syringe is connected by Tygon tubing to a single-channel fluid swivel (375 Series Single Channel Swivels, Plymouth Meeting, PA) which was mounted on a balance arm above the chamber.

The effects of the TLR4 antagonists (+)-naloxone and (+)-naltrexone are being tested on self-administration of the μ-opioid agonist, remifentanil. Remifentanil was chosen for testing rather than morphine due to its very short half-life which promotes high rates of self-administration and stability of lever pressing. Rats are first trained on cocaine self-administration, as previously described (Hiranita et al., 2009), and are subsequently tested with remifentanil substituted for cocaine. Remifentanil injections reliably maintain self-administration at high rates that are dependent on dose of drug. From our past studies, and from early work on this study to date, we find an inverted U-shaped dose-effect curve for remifentanil; this shape of the dose-effect curve is characteristic of that for other drugs of abuse. The highest rate of responding in our experience is maintained at a dose of 1.0 μg/kg/injection, with lower response rates at higher and lower doses. Response rates in our experience are

affected by remifentanil dose, with rates maintained by 1.0 μg/kg/injection of remifentanil being greater than those obtained when responses had no consequences (EXT). To date in our initial animals for this project, the same results for the basic remifentanil effects are being replicated.

We have initiated the studies of (+)-naloxone and (+)-naltrexone. While early, the initial data are suggestive that an attenuation of remifentanil self-administration is a likely result. Significant progress on this project is anticipated in the coming year.

To define specificity of effects, we have undertaken the study of a separate group of subjects trained with food reinforcement. This is being done in order to assess the specificity of the effects of (+)-naloxone and (+)-naltrexone on remifentanil self-administration as an affect on food reinforcement would basically change interpretation of results of TLR4 blockade for opioids or cocaine. Hence this control study is applicable to the cocaine aspects of this project as well, so is especially worthwhile to undertake. Experimental procedures were identical to those detailed above except that each completion of five responses delivered a food pellet rather than an injection of remifentanil. The selectivity of the effects of (+)-naloxone and (+)-naltrexone were assessed by comparing the effects at the maximal rates of responding maintained by either the dose of remifentanil or the amount of food that maintained the highest rate of responding. This study is ongoing.

Task 5. Aim 2B. Blockade of reinstatement by systemic (+)-naltrexone.

This study has recently begun. Sprague-Dawley rats (Taconic Farms, Germantown, New York) weighing approximately 300 g at the start of the study, serve as subjects. Subjects are acclimated to a temperature- and humidity-controlled vivarium for at least one week with a 12:12-h light:dark cycle (lights on at 07:00 hours) during which food and tap water are available at all times. After acclimation, body weights are maintained at approximately 320 g by adjusting the daily food ration with water remaining available at all times in the home cages. Subjects in studies of food reinforcement are fed their daily food ration (~35 g of 1-g chocolate-flavored pellet, Bio-Serv) 150 min before sessions, so that their response rates approximated those maintained by drug injections.

Care of the subjects was in accordance with the guidelines of the National Institutes of Health and the National Institute on Drug Abuse Intramural Research Program Animal Care and Use Program, which is fully accredited by AAALAC International.

Experimental sessions are to be conducted daily with subjects placed in operant-conditioning chambers (modified ENV-203, Med Associates, St. Albans, VT) that measured 25.5 x 32.1 x 25.0 cm that are enclosed within sound-attenuating cubicles equipped with a fan for ventilation and white noise to mask extraneous sounds. On the front wall of each chamber are two response levers, 5.0 cm from the midline and 4.0 cm above the grid floor. A downward displacement of either lever with a force approximating 0.20 N defines a response, and always activates a relay mounted behind the front wall of the

chamber producing an audible “feedback” click. Six light-emitting diodes (LEDs, three yellow and three green ones) are located in a row above each lever. A house light is located at 25 cm above the grid floor (near the ceiling) at the center of the front wall of the chamber. A receptacle for the delivery of 45-mg food pellets via a pellet dispenser (Med Associates, Model ENV-203-20), is mounted on the midline of the front wall between the two levers and 2.0 cm above the floor. A syringe infusion pump (Model 22, Harvard Apparatus, Holliston, MA) placed above each chamber delivers injections of specified volumes from a 10 ml syringe. The syringe is connected by Tygon tubing to a single-channel fluid swivel (375 Series Single Channel Swivels, Plymouth Meeting, PA) which is mounted on a balance arm above the chamber.

For (-)-heroin self-administration, subjects are surgically prepared under anesthesia (ketamine 60.0 mg/kg, i.p. and xylazine 12.0 mg/kg, i.p.) with a chronic indwelling catheter in the right external jugular vein. The catheter exits the subject at the mid-scapular region of its back. Catheters are infused daily with 0.1 ml of a sterile saline solution containing heparin (30.0 IU/ml) and penicillin G potassium (250,000 IU/ml) to minimize the likelihood of infection and the formation of clots or fibroids. All animals are allowed to recover from surgery for approximately seven days before drug self-administration studies were initiated.

Tygon tubing from the swivel to the subject’s catheter is protected by a surrounding metal spring and completed the connection to the subject.

We plan to run food reinforcement as well as heroin to assess conditions under which reinstatement can be reliably obtained. Once those conditions are established, these experimental conditions will be used to assess the specificity of (+)-naltrexone on reinstatement of heroin self-administration. Effects of (+)-naltrexone will be compared on reinstated responding previously reinforced with food and, in a separate group as detailed below, on reinstated responding previously reinforced with heroin injections.

Experimental sessions start with the illumination of the house light and yellow LEDs above each lever. The house light remains illuminated throughout sessions except during each timeout period between components. Each completion of five responses (FR5) on the right lever turn off the green LEDs, turn on the yellow LEDs for four sec, and deliver a food pellet or activate the infusion pump for i.v. delivery of (–)-heroin (0.01 mg/kg/injection). A 20-sec time-out period follows, during which responding has no scheduled consequences (time out). After the time out, the green LEDs are illuminated and food or (–)-heroin injections are again available under the FR5 schedule. Responses on the left lever are recorded but have no scheduled consequences. Training continues until response rates are stable from one session to the next.

Once performances are stable, reinstatement test sessions are conducted. These test sessions are similar to training sessions except that for subjects trained with food reinforcement, the daily food regimen is withheld. For both subjects trained with food reinforcement and those trained with heroin injections, responses during the first component have no scheduled consequences (extinction). Reinstatement is assessed in the remaining four

components. For subjects trained with food reinforcement, those components after the first are preceded by the response-independent presentations of from one to four 20-mg food pellets. For subjects trained to self-administer heroin, each of the subsequent four components assess the reinstatement after response-independent infusions of heroin at doses from 1.0 to 32.0 µg/kg. In other tests with subjects trained with heroin, the effects of response-independent infusions of saline (5.6, 18.0, 56.0, and 180 µl) will be assessed. Finally, the effects of 32 mg/kg of (+)-naltrexone are assessed on the reinstatement induced by heroin doses of 1.0 to 32.0 µg/kg.

This study has been initiated and is ongoing. Significant progress is anticipated in the coming project period now that basic procedures are worked out.

Conclusions

This project is progressing excellently and remains on schedule across research sites as defined by the Statement of Work. From studies completed to date, our major findings are that (a) morphine-induced increases in extracellular dopamine in the Nucleus Accumbens (NAc) shell is suppressed by microinjection of the toll like receptor 4 (TLR4) antagonist LPS-RS into either the NAc or the ventral tegmental area (VTA). Intriguingly, suppression of NAc dopamine in response to cocaine is also observed by NAc or VTA LPS-RS. This is exciting as it challenges the broadly held view that the major effect of cocaine is on dopamine transporters in the VTA. Given this intriguing result, a control study was undertaken to ensure specificity of the results obtained. Specificity was observed since LPS-RS had no effect on dopamine released in response to intra-VTA neurotensin. Multiple additional Aims and SubAims have begun and significant progress anticipated in the coming project period.

Key Research Accomplishments

* One manuscript is in preparation for publication and expected to be submitted for review near term

* The work has been presented as part of multiple invited research seminars by the PI at conferences and universities

Reportable outcomes

* The work is part of Alexis Northcutt's ongoing PhD dissertation research project

* One manuscript is in preparation for publication and expected to be submitted for review near term

The work has been presented as part of multiple invited research seminars by the PI at conferences and universities

Conclusions

We are making excellent progress and have maintained the required outputs and data collection according to the statement of work. Toll like receptor 4 (TLR4) continues to present as a powerful modulator of drug abuse as blocking TLR4 with (+)naltrexone or (+)-naloxone suppresses multiple indices of drug reward and drug reinforcement. It is exciting that we are documenting the sites of action of TLR4 on drug abuse to the NAc shell and VTA, key structures in the rewarding and reinforcing effects of cocaine and opioids, the abused drugs under study. As (+)-naltrexone is in preclinical development aiming at human clinical trials, this is especially exciting to have a blood brain barrier permeable, highly selective TLR4 antagonist that would be orally available, stable at room temperature, and appropriate for use from front lines through long-term use. We are awaiting the decision on a requested grant proposal from the Army which would move (+)-naltrexone to FDA Investigational New Drug status ready for entry into human clinical trials. Given our ongoing results, this would be a spectacular step forward for treating warfighters and veterans alike for drug abuse indications.

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